Review Article

Predicting the Transfer of Contaminants in Ruminants by Models – Potentials and Challenges

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Abstract

Undesirable substances in feed can transfer into foods of animal origin after ingestion by livestock animals. These contaminants in food may threaten consumer health. Commonly, feeding trials are conducted with animals to assess the transfer of undesirable substances into animal tissues or milk. Such feeding trials explore the effects of the various physiological systems (e.g., ruminant and non-ruminant gastro-intestinal tracts) as well as different livestock production intensities on transfer. Using alternative methods to mimic the complex physiological processes of several organs is highly challenging. This review proposes a potential cascade of *in vitro* and *ex vivo* models to investigate the transfer of contaminants from feed into foods of animal origin. One distinct challenge regarding the models for ruminants is the simulation of the forestomach system, with the rumen as the anaerobic fermentation chamber and its epithelial surfaces for absorption. Therefore, emphasis is placed on *in vitro* systems simulating the rumen with its microbial ecosystem as well as on *ex vivo* systems to replicate epithelial absorption. Further, the transfer from blood into milk must be evaluated by employing a suitable model. Finally, *in silico* approaches are introduced that can fill knowledge gaps or substitute *in vitro* and *ex vivo* models. Physiologically-based toxicokinetics combines the information gained from all alternative methods to simulate the transfer of ingested undesirable substances into foods of animal origin.

1 Introduction

Expectations of animal-derived food are high. Besides being available in sufficient quantities, animal-derived food is expected to be of high nutritional value, while food safety risks to consumer health must be low. Undesirable substances in feed can pose a threat to consumer health if, upon ingestion by livestock, they are transferred into foods of animal origin, where they are then considered contaminants.

"Undesirable substances" in EU legislation on animal nutrition means any substance or product, except for pathogenic agents, present in and/or on the product intended for animal feed that presents a potential danger to human health, animal health or the environment or adversely affects livestock production (Directive 2002/32/EC). In comparison, "contaminants" are substances that have not been intentionally added to but may be present in food as a result of the various stages of its production, packaging, transport or storage. They also might result from environmental contamination (Council Regulation (EEC) No 315/93). Accordingly, undesirable substances are regulated for feed, while contaminants are regulated for food.

REACH (Regulation (EC) No 1907/2006) requires, according to Article 14(4), exposure assessment and subsequent risk characterization to be carried out for chemical substances subject to registration that are manufactured or imported in quantities equal to or greater than 10 (metric) tons per year and where the substance fulfils the criteria for any of the hazard classes or categories indicated in Article 14(4)2 or is assessed to be persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB). As such, for substances in plant protection products (Council Directive 91/414/EEC) as well as for the authorization of several feed additives (Commission Regulation (EC) No 429/2008), investigations on a possible transfer from feed to food are mandatory to establish withdrawal periods, maximum residue levels (MRLs) or maximum contents in feedstuffs (Directive 2002/32/EC). This routinely requires feeding

Received July 8, 2020; Accepted December 11, 2020;
Epub December 16, 2020; © The Authors, 2021.

ALTEX 38(3), 398-418. doi:10.14573/altex.2007081

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trials with various livestock species to cover the different physiological systems (e.g., ruminant gastro-intestinal tract (GIT) and non-ruminant GIT). These *in vivo* trials are time-consuming, expensive, and often require killing of the animals to harvest organs and tissues for contaminant analyses (e.g., according to OECD TG 503 and OECD TG 505; OECD, 2007a,b). Following the 3R principle (replace, reduce, refine) introduced by Russel and Burch (1959), alternative models (*in vitro*, *ex vivo*, *in silico*) are being developed and tested to simulate the relevant *in vivo* processes as closely as possible. To employ them for regulatory purposes, their development needs to undergo a thorough validation to ensure scientific integrity and quality (OECD, 2018).

Various partial gastro-intestinal *in vitro* and *ex vivo* systems to evaluate the nutritional value of feedstuffs already were established decades ago. These can simulate the process of nutrient digestion and absorption in the GIT. However, so far, they have been used only rarely to study the transfer and metabolism of undesirable substances in the animal. Moreover, to investigate potential substance transfer into animal tissues or excreta when ingested with feed, the physiological processes downstream need to be simulated by a cascade of individual methods to capture the whole complexity of animal metabolism. Simulating processes in the ruminant is especially challenging due to the physiological distinctiveness of the forestomach system and the highly different metabolic statuses the animal can be in during its life, e.g., dairy cows during the onset of lactation vs. beef cattle.

Recently introduced *in silico* models may help to simulate the interactions between individual body compartments as well as species-specific differences. The decision on whether non-animal methods are suitable for reproducing the metabolism of an undesirable substance in the animal requires a detailed examination of the functioning, potential, and limitations of each individual method (Tab. 1).

The present report reviews *in vitro* and *ex vivo* models that simulate the physiological processes in ruminant species, beginning with the complex forestomach system, including intestinal absorption and hepatic metabolization, and ending in excretion via milk. The focus is on the fate of foreign substances in the organism. In this context, the fate is called kinetics and the substances xenobiotics. An *in silico* approach of kinetic modelling is introduced to model the interactions between the individual *in vitro* and *ex vivo* compartments to complete the understanding of the entire animal metabolism and close the gap between *in vitro* and *in vivo* results.

2 *In vitro* reticulo-ruminal digestion and fermentation

2.1 General considerations regarding ruminant species

Ruminants are unique in their mode of plant digestion, as they have evolved a forestomach, which allows microbial digestion and fermentation of fibrous carbohydrates, i.e., celluloses and hemicelluloses, leading to the formation of short chain fatty acids (SCFA), which constitute the major energy source for the ruminant (Bergman, 1990). The ruminant's stomach system is composed of the reticulum, rumen, omasum, and abomasum. The reticulum and rumen are joined by a fold of tissue and constitute a large fermentation chamber that houses a highly diverse rumen microbiome with manifold enzymatic activities (Moraïs and Mizrahi, 2019). The rumen wall is covered by a keratinizing stratified squamous epithelium in the form of countless papillae for effective absorption and secretion via a permeation barrier (Aschenbach et al., 2019). The reticulum is further connected to the omasum, also called manyplies because of the numerous parallel sheets of tissue, which serves as a bottleneck for feed particle passage from the reticulo-rumen. Fifteen percent of the water that enters the omasum is absorbed here (Krehbiel, 2014). The following abomasum is like the non-ruminant stomach, containing a glandular gastric mucosa with specialized secretory cells that produce mucus, pepsinogen, and hydrochloric acid.

For decades, experiments have been conducted to compare ruminant species' intake and digestive capacity by feeding a wide range of diets with different ingredients and chemical compositions. In a meta-analysis, Riaz et al. (2014) stated that numerous studies have compared feed intake and nutrient digestibility between sheep and goats and between sheep and cattle, fewer studies have made comparisons between cattle and buffaloes, and even fewer studies have been published on the comparison of feed intake and digestibility among more than two ruminant species. These comparative studies indicate that there are differences between ruminant species regarding both intake and digestive capacity at the reticulo-ruminal and total digestive tract level and that additional differences between genotypes, e.g., between Jersey versus Holstein-Friesian cows, must be considered (Beecher et al., 2014).

However, others found no systematic differences between sheep and cattle in digesting maize silage and grassland products (Aerts et al., 1984). Moreover, interspecies differences in many studies were, in part, related to differences in the passage rates of solids (Bartocci et al., 1997) or fluid (Colucci et al., 1990) or both (Francoise Domingue et al., 1991; Poppi et al., 1980) from the reticulo-rumen. It may therefore be argued that reticulo-ruminal *in vitro* systems, where reticulo-ruminal retention times or the reciprocals, i.e., outflow rates, are constantly controlled regardless of ruminant species, are less prone to be affected by species differences than *in vivo* studies. Therefore, the following considerations will focus on studies that have compared ruminal contents from different species in *in vitro* experiments.

More than 30 years ago, Kudo et al. (1984) showed that the toxic amino acid mimosine was degraded to a similar extent *in vitro* in ruminal fluid from both cattle and sheep and that the degradation occurred faster when the donor animals were fed a mixed forage-concentrate diet compared to forage alone. When grass silage or maize silage were incubated in a rumen simulation technique (RUSITEC) system using ruminal contents from either cows or sheep fed on three different diets, Boguhn et al. (2013) observed that *in vitro* nutrient degradation and microbial crude protein synthesis were more affected by the diet of the donor animals than by the animal species and was probably mediated by an adjusted microbial community. This was confirmed by Witzig et al. (2015), who analyzed the composition of the microbial community in the

Tab. 1: Pros and cons of the reviewed *in vitro*, *ex vivo* and *in silico* methods proposed for investigating the transfer of undesirable substances from feed to food

Technique	Simulated or replaced organ or tissue	Pros	Cons
Continuous culture models	Rumen, colon, caecum	– No killing of animals needed. – Long term incubation allows investigation of bacterial adaptation to xenobiotics.	– Donor animals needed; one-time surgery required. – Cannulated animals need extra care. – Loss of ruminal protozoa – No epithelial absorption and secretion – Time intensive – Apparatus is commonly not commercially available.
Batch culture	Rumen, colon, caecum	– No killing of animals needed. – Easy and fast – Allows simultaneous investigation of several substances or dosages.	– Donor animals needed; one-time surgery required. – Cannulated animals need extra care. – No epithelial absorption and secretion – Short term incubation likely does not allow microbial adaptation to incubation conditions and xenobiotics.
Ussing chamber	All gut segments	– Different gut segments can be tested simultaneously.	– Animals must be killed. – Viability of tissues is limited. – Costly and laborious
Immortal epithelial cell lines	Rumen, small intestine	– No killing of animals needed. – Rapid screening of various contaminants, their concentration and interaction – Allows studying mechanisms at cellular level.	– Sometimes different physiological behavior compared to *in vivo* conditions. – No/limited interaction with other cell types (e.g., immune cells) – Missing mucus layer – Results are valid on cell level but cannot necessarily be extrapolated to complex *in vivo* conditions.
Liver S9 mix and microsomes	Liver, intestine, kidney, lung, skin	– Reduced number of animals as organ donors needed. – High ethical acceptance – Cheap and easy to produce. – S9 contains both membrane-bound and cytosolic liver enzymes. – Phase I and phase II reactions can be specifically investigated with the addition of appropriate cofactors. – Available from all relevant metabolizing organs.	– Microsomes contain only membrane-bound enzymes. – Addition of cofactors required. – Investigation of transport mechanisms is not possible. – Comparability to the *in vivo* situation is lower than for immortalized and primary cell lines.
Organ perfusion models	Liver, kidney, udder	– Complex organ cellular interaction – Active *in vivo*-like transport and metabolism conditions – Better understanding of quantitative contribution of the organ to certain processes	– Animals must be killed. – Viability of tissues is limited. – Ischemia/reperfusion-caused cellular stress/damage
Toxicokinetic modelling	All organs and tissues	– No killing of animals needed. – Can generate predictions of future scenarios. – Extrapolation to different species, chemicals and settings is possible. – Cheaper than animal experiments	– Reliance on extensive datasets – Some data needs to be generated by animal experiments (for now).

RUSITEC experiment of Boguhn et al. (2013) and found that the effect of donor animal species was limited to the number of archaea, which was greater for sheep than for cows. These authors concluded that the rumen microbial community that establishes *in vitro* is primarily affected by the donor animal's diet. Based on their findings, the authors suggested using a standardized approach for studying the rumen microbiota in a rumen simulation. This suggestion was underlined in a recent study (Belanche et al., 2019), in which the authors emphasized that collection time, donor animal diet, fermentation substrate, and inoculum preservation method may all have an impact on the study variables.

In accord with the observations outlined above, Henderson et al. (2015) reported that differences in ruminal microbial community compositions were predominantly determined by the diet and much less by the species. Moreover, a core microbiome was found across geographical regions and ruminant species, such that similar bacteria and archaea dominated in nearly all samples, and only protozoal communities were more variable. Accordingly, the "core bacterial microbiome" at the genus or higher levels comprises *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales*, providing a multitude of enzymes needed for the breakdown of nutrients (Hartinger et al., 2018; Huws et al., 2018; Moraïs and Mizrahi, 2019).

In summary, it appears that ruminal contents from different ruminant species are equally suitable for *in vitro* studies on rumen metabolism, provided that feeding management of animals and handling of ruminal fluid is standardized and procedures are applied consistently, regardless of the origin of ruminal fluid.

2.2 Continuous culture models

The rumen harbors a complex anaerobic ecosystem consisting of different niches of microbes, which enable extensive degradation and fermentation of feedstuffs. Diverse microbes have been shown to either adhere to fiber particles, be associated with the liquid phase (McAllister et al., 1994; Klevenhusen et al., 2017) or be attached to the rumen epithelium (Petri et al., 2013; Wetzels et al., 2016). Accordingly, *in vitro* systems simulating the rumen fermentation should ideally preserve this complex microbial community, mirroring the original rumen microbiota and fermentation processes, and therefore the *in vitro* fermenters must be inoculated with the original ruminal microbiota from donor animals.

Ruminal contents for inoculation can be harvested either from slaughterhouses or from ruminally cannulated animals. The advantages of using ruminally cannulated animals are that their medical history is known and their feeding management can be controlled and adapted to specific research questions. Cannulated animals are commonly kept in research facilities with close access to the laboratories for *in vitro* incubation. Short transport distances and times ensure microbial survival. In comparison, it is often difficult to find slaughterhouses near research facilities, and therefore transport distances are often too long to maintain the ruminal microbiota. Although ruminally cannulated animals can live long, healthy lives, the cannulation itself is a surgical intervention considered to be an animal experiment and thus requires sound justification.

Several continuous culture systems were developed in the 1960s (Aafjes and Nijhof, 1967), however their applicability was often limited. Three major systems are routinely used in several laboratories worldwide today: the rumen simulation technique (RUSITEC), which was established in the 1970s by Czerkawski and Breckenridge (1977, 1979a,b); a system first described by Slyter et al. (1964), in which solid feed is added directly to the fermenters and the overflow is collected; and the dual flow continuous culture system designed by Hoover et al. (1976), in which solid feed is also added directly to the fermenters but the overflow of the liquid phase and the solid phase leave the fermenters separately with different outflow rates.

The RUSITEC system typically comprises 4 to 8 fermenters, each having a volume of 700 to 1000 mL. Artificial saliva (e.g., McDougall, 1948) is infused continuously at the bottom of the vessel, and the overflow of the liquid phase and the fermentation gases are forced through an outflow by a small positive pressure in the gas space. The overflow and the produced gas can be collected in bottles and gastight bags, respectively, for quantification and analysis. Degradability of the diets is commonly determined from synthetic fiber feed bags after 48 h of incubation. Pore sizes of the feed bags vary between studies, from 50 to 200 µm.

On the first day of a RUSITEC experiment, each fermenter is filled with a mixture of rumen fluid from at least three donor animals, as proposed by Udén et al. (2012), and artificial saliva. In addition, approximately 80 g solid material of the rumen fiber mat (Czerkawski and Breckenridge, 1977) is provided in a feed bag to enable the establishment of both liquid- and particle-associated ruminal microbes. An additional feed bag contains the experimental diet to ensure nutrient delivery to the microbes. Both bags are placed into the "food container" inside of each fermenter, which is continuously moved up and down to warrant constant mixing of the fluid and the solid phase (Czerkawski and Breckenridge, 1977). Feed bags are usually incubated in the fermenters for 48 h before being replaced with fresh ones.

In routine RUSITEC experimental runs, the digestibility of different feedstuffs or diets as well as fermentation variables such as the production of SCFA and methane can be determined in the relatively short time of 10 to 15 days (e.g., Hindrichsen et al., 2004; Khiaosa-Ard et al., 2009; Terry et al., 2018); however, longer experiments of more than 20 days also have been successfully conducted (e.g., Wallace and Newbold, 1991; Soliva et al., 2004). To obtain statistically valid results, sufficient replicates need to be obtained by repeating the experimental runs, thus prolonging the full experiment to several weeks.

One limitation of the RUSITEC system is that, although bacterial populations can be largely maintained for several days and even weeks (Ziemer et al., 2000; Wetzels et al., 2018), the abundance of protozoal populations has been shown to decrease considerably after a few days (Ziemer et al., 2000; Martínez et al., 2010b; Lengowski et al., 2016). This is likely a result of the high liquid turnover rate in comparison to the generation intervals of the protozoa (Potter and Dehority, 1973).

Single and dual flow continuous culture systems as proposed by Slyter et al. (1964) and Hoover et al. (1976) on the other hand have been shown to also maintain the protozoal populations (Mi-

ettinen and Setälä, 1989), albeit this strongly depends on the turnover rate and stirring conditions applied (Mansfield et al., 1995; Moumen et al., 2009). Hoover et al. (1976) designed a dual effluent removal system to simulate the differential flows for liquids and solids found in the rumen, allowing a longer residence time for the digestion of solid particles. A variable rate mechanical feeding device apportions the feed into the fermenter at regular intervals during the day. A magnetic stirrer operates intermittently, stirring the contents for 5 min every 5 h at low speed to minimize possible deleterious effects of mechanical agitation on the protozoa. However, unlike the RUSITEC system, which is commonly inoculated with ruminal fluid and solid material from the ruminal fiber mat, the systems of Slyter et al. (1964) and Hoover et al. (1976) are inoculated only with ruminal liquid. Thus, although protozoa might survive for a longer period and in greater abundance, ruminal microbes attached to the fiber mat might be generally missing or are at least underrepresented.

Continuous culture approaches are often used to investigate dietary effects and effects of feed additives on fermentation characteristics, such as nutrient degradation and methane formation (e.g., Soliva et al., 2004; Busquet et al., 2005; Terry et al., 2018; Petri et al., 2019). However, so far only few studies have used any of the described continuous culture techniques to investigate the ruminal degradation or biotransformation of undesirable substances. For instance, researchers analyzed the effects of *Fusarium* toxins on nutrient utilization, the turnover of deoxynivalenol and zearalenone (Seeling et al., 2006), and the responses of the rumen microbial communities to *Fusarium*-contaminated feed (Strobel et al., 2008; Boguhn et al., 2010) with RUSITEC. They could show that deoxynivalenol and zearalenone were only slightly metabolized by the *in vitro* rumen microbiota. Koch et al. (2006) demonstrated alterations in the microbial population structures and their metabolic profiles in the presence of transgenic maize, while application of a glyphosate-containing herbicide in concentrations reflecting potential exposure of dairy cows or beef cattle did not exhibit significant effects on bacterial communities in RUSITEC (Riede et al., 2016). Kowalczyk et al. (2015) studied the recovery of perfluoroalkyl acids during the incubation of contaminated feed in RUSITEC. Recently, Birk et al. (2018) modified the RUSITEC system to allow testing ^{14}C-labelled metabolites of azole fungicide and thus showed that the system can also be used to work with radiolabeled test compounds.

One reason for the lack of more studies might be the relatively long duration of a complete experiment to achieve a reasonable number of replicates for statistical evaluation. Other reasons might be the change in microbial populations and community structure with prolonged time of incubation and the constant dilution by buffer infusion. Accordingly, short term batch culture approaches might be easier to conduct and more appropriate to investigate microbial effects on the fate of contaminants in ruminal fluid (see Section 2.3.).

Although continuous culture approaches can maintain a steady fermentation pattern, which is very similar to the pattern observed in the rumen of the donor animals (Hannah et al., 1986; Mansfield et al., 1995; Martínez et al., 2010a) over several days up to weeks, it remains to be answered if the *in vitro* degradation and biotransformation of contaminants from feeds fully represent *in vivo* microbial processes, as the epimural microbiota, which is associated with the ruminal epithelium, is missing *in vitro*, and fiber-attached microbes (i.e., bacteria and fungi) and protozoa are underrepresented. Nevertheless, continuous culture approaches can provide information on whether certain contaminants are likely to be transformed by ruminal microbes or not.

Besides the epimural microbiota, it should be stressed that so far none of the developed incubation systems include a simulation of the ruminal epithelium with its capacity for absorption of fermentation acids and possible harmful substances as well as its release of signal molecules into the rumen. The *in vitro* fermentation systems do not include a provision for removal of soluble substances except through the overflow. Accordingly, additional methods to investigate epithelial absorption processes are needed as outlined in Section 3.

2.3 Batch culture techniques

In vitro batch culture systems based on ruminal fluid have traditionally been used to estimate (total-tract) organic matter digestibility, and standardization and routine application of these methods was discussed already more than 50 years ago (e.g., Alexander and McGowan, 1966). Of all *in vitro* batch culture systems, gas production techniques have attracted the attention of researchers to study not only ruminal feed digestibility, but direct or indirect impacts of animal production on the environment (Krishnamoorthy et al., 2005). This widespread attraction became notably evident in a comprehensive special issue published in *Animal Feed Science and Technology* in 2005, comprising 40 publications (Krishnamoorthy et al., 2005). Topics covered in reviews and research papers, as outlined in the preface to the special issue by Krishnamoorthy et al. (2005), comprised methodologies, repeatability, application to feed nutritive evaluation and feed secondary compounds, application to investigate environmental impacts such as methane production, application to simple-stomached animals and humans, describing and predicting gas production, and quantitative possibilities for gas data. Application of a range of *in vitro* batch culture systems, including gas production techniques, to nutritive evaluation of food in the hindgut of humans and other simple-stomached animals was also reviewed at that time (Coles et al., 2005), and it was pointed out that, compared with techniques simulating ruminal events, *in vitro* digestion methods of relevance to human food evaluation lack standardization as well as *in vivo* validation and justification.

Generally, *in vitro* gas production techniques are versatile tools to study ruminal digestion and microbial metabolism, although they also have limitations (Rymer et al., 2005). More recently, Yáñez-Ruiz et al. (2016) reviewed the applicability of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants and provided a range of technical recommendations to harmonize techniques for feed evaluation and assessment of rumen function and methane production. The wide applicability of *in vitro* gas production techniques to study ruminal events is also reflected in modifications of the method, where ruminal fluid is

replaced with buffered fecal suspensions from ruminants (Aiple et al., 1992) and other herbivores (e.g., horses; Can et al., 2009), which would allow replacing ruminally fistulated animals with intact animals.

Numerous batch culture studies have been conducted to screen the effects of bioactive substances (e.g., plant secondary metabolites) on microbial activity (e.g., methane formation; reviewed by Lewis et al., 2013; Morgavi et al., 2010; Yáñez-Ruiz et al., 2016) and fermentation kinetics. However, like with the continuous culture approach, only few studies have been carried out to determine the effects of undesirable substances or contaminants, and those few studies focused mostly on fungal metabolites. For example, Asiegbu et al. (1995) and Mojtahedi et al. (2013) investigated the effects of aflatoxins, and Jeong et al. (2010) studied the effects of deoxynivalenol on *in vitro* gas and SCFA production. Morgavi et al. (2013) explored the methane inhibiting effects of fungal secondary metabolites from *Monascus spp*. using a batch culture approach, and Akkaya and Bal (2012) determined the aflatoxin binding capacities of a *Saccharomyces cerevisiae* extract and mycotoxin adsorbents based on hydrated sodium calcium aluminosilicate. Using batch culture, Hahn et al. (2015) tested whether 20 commercially available products could detoxify deoxynivalenol and zearalenone.

Even fewer studies investigated whether the rumen microbial activity degrades or biotransforms a substance of interest. Caloni et al. (2000) demonstrated a low depletion rate of Fumonisin B_1 in batch culture, and Mobashar et al. (2012) quantified the microbial degradation of ochratoxin A using the Hohenheim gas test, distinguishing the degradation efficiency of individual microbial groups by applying antibiotics and fungicides. Likewise, by using a centrifugation protocol, Westlake et al. (1989) and Kiessling et al. (1984) demonstrated the degradation of several mycotoxins by bacterial and protozoal preparations from ovine ruminal fluid.

However, although a quick degradation of toxic compounds can sometimes be measured in batch culture, this does not always fit the observed fermentation pattern. For example, although patulin was shown to be unstable in rumen contents, decreasing to 50% after 4 h of incubation and being hardly detectably after 18 h, it was still highly toxic to *in vitro* rumen fermentation (Morgavi et al., 2003). The same group also found that gliotoxin, a mycotoxin often found in conserved forages, was unstable in the rumen environment, decreasing by 90% after 6 h of incubation (Morgavi et al., 2004). Others, though, demonstrated the stability of some mycotoxins in the *in vitro* rumen. As such, recoveries of mycophenolic acid and roquefortine C, both mycotoxins of *Penicillium* section *roqueforti*, were 79% and 41%, respectively, after 48 h of incubation (Gallo et al., 2015). Likewise, aflatoxin B_1 and deoxynivalenol were not degraded by ruminal microbes *in vitro* (Kiessling et al., 1984). Apparently, these compounds are quite resistant to microbial degradation or ruminal biotransformation and likely reach the lower gut.

Only few studies have investigated contaminants other than fungal metabolites in batch culture. For example, Majak and Cheng (1987) determined the rates of glycoside hydrolysis and hydrogen cyanide release of three different cyanogenic glycosides, and de Oliveira et al. (2010) investigated the ruminal degradation of the toxic protein ricin found in castor seed. Some countries have reported contamination of milk by heavy metals like lead (Pb). Nurdin and Susanty (2015) used a batch culture approach to test the Pb binding efficacy of herbal additives in the rumen. They showed that less dissolved Pb was found in the ruminal fluid after incubation with *Cuminum zedoaria*, *Curcuma mangga* and *Cuminum cyminum*, suggesting a possible transfer of bound Pb to the lower gut and excretion with the feces. Using *in vitro* batch culture, Váradyová et al. (2006) showed that inocula of sheep that grazed on pastures contaminated with heavy metals resulted in significantly lower fermentation output than a control inoculum. Also, Craig et al. (1992) demonstrated the microbial degradation of pyrrolizidine alkaloids using ovine rumen fluid.

In summary, batch culture studies can be used to determine the degree of contaminant degradation or biotransformation and the contaminants' effects on ruminal microbes and ruminal fermentation. Consequently, results from batch culture studies can significantly help as a decision support for follow-up investigations. They are easier and faster to apply than continuous batch culture approaches, resulting in larger numbers of replicates. However, continuous culture studies might be advantageous to determine whether longer term microbial adaptation towards certain substances can occur.

3 Models for the intestinal transfer of nutrients and contaminants

The different segments of the gastrointestinal tract differ substantially within and between species regarding their anatomy and physiological function, but the epithelium lining the intestinal tract represents the first and major barrier for both nutrients and contaminants throughout.

The Ussing chamber technique is the only experimental approach for studying transepithelial transport processes that allows quantification of unidirectional flux rates of molecules across the epithelial barrier as well as the electrophysiological characterization of transport processes. It also allows studying xenobiotic metabolism by intestinal epithelial cells through targeted or non-targeted analysis of xenobiotic metabolites. This technique originally was introduced by the Danish physiologist Hans Ussing to measure ion transport processes across frog skin (Ussing, 1949; Ussing and Zerahn, 1951).

Intestinal segments are taken immediately after slaughter, rinsed with physiological saline at 4°C and opened longitudinally. The tunica mucosa is stripped of the muscular and serosal layer, and the mucosal tissue is then mounted between the two halves of an Ussing chamber with an exposed surface of up to 2 cm^2, thus forming a mucosal and a serosal compartment. Each side of the chamber is connected to a buffer reservoir, which is continuously gassed with carbogen (95% O_2, 5% CO_2). An isotonic buffer solution with a pH of 7.4 commonly is used on each side to mimic physiological conditions. To maintain the viability of the epithelial tissues, glucose is added at a concentration of

10 mmol/L to the serosal buffer solution used for incubating tissues from the small intestines. For hindgut tissues, the mucosal buffer solution should contain acetate, propionate and butyrate at physiological molar proportions with an overall concentration of 60 mmol/L.

The Ussing chamber is connected to a computer-controlled voltage clamp unit. Electrodes located close to the tissues continuously measure the transepithelial potential difference (PD_t). Under open circuit conditions, defined currents, which induce a short-term change in PD_t, are applied to the tissue at regular intervals. The transepithelial tissue resistance can be calculated using Ohm's law. Active transepithelial electrogenic transport processes generate an electric current that can be set to zero by introducing a respective short circuit current (I_{sc}) by a further pair of electrodes. Under these conditions, the I_{sc} is a measure for all electrogenic transport processes. When both chemical and electrical gradients are eliminated, the transport properties can be determined by measuring unidirectional flux rates from the mucosal to the serosal (J_{ms}) and from the serosal to the mucosal (J_{sm})side of the tissue using radioactively labelled substrates. In the absence of any electrochemical gradient, the unidirectional flux rates differ significantly and result in a significant net flux (J_{net}) when active processes are involved in either absorption or secretion.

Numerous studies have been carried out in recent years to identify the transport properties of intestinal phosphate (reviewed by Muscher-Banse and Breves, 2019) and gastrointestinal calcium in ruminants (Wilkens et al., 2012; Schröder et al., 2015). The effect of SCFA on electrophysiological and co-transport properties of calcium, sodium or urea across the bovine and ovine rumen epithelium have been studied, respectively (Sehested et al., 1995; Uppal et al., 2003; Abdoun et al., 2010). The Ussing chamber technique has also been used to understand chloride secretion and intraepithelial metabolism of histamine in both the porcine colon and the bovine rumen (Aschenbach and Gäbel, 2000; Ahrens et al., 2003; Kröger et al., 2013, 2015). Whereas the flux rates of electrolytes can be quantified based on radioactively labelled substrates, the exact measurement of organic nutrients or contaminants necessitates a more laborious approach, as most organic compounds are subjected to intraepithelial metabolism. Thus, sensitive analytical methods are needed to quantify the mucosal uptake and serosal release, also of metabolites formed in the epithelium, and the potential tissue accumulation as a function of time. This approach was successfully introduced for measuring transport processes of SCFA in the porcine hindgut (Herrmann et al., 2011). In addition, epithelial transfer of the colon carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) was evaluated across the rat intestine (Nicken et al., 2013). Finally, the Ussing chamber technique has been successfully applied to measure drug transfer in human intestinal tissues (Rozehnal et al., 2012; Sjöberg et al., 2013). Alternatively, the specific radioactivity in the mucosal and serosal compartments can be measured.

The gastrointestinal segments can only be incubated for limited times. Whereas rumen epithelial tissues can be incubated for at least 6-7 h, tissues from the small intestine can only be used for approximately 2.5-3.5 h; hindgut tissues survive for intermediate times. A viability test must be performed after each experiment using secretagogues such as forskolin, theophylline or carbachol or nutrients that are transported by electrogenic mechanisms to confirm tissue functionality during the experiment.

Other *in vitro* incubation techniques such as the everted sac technique (Harmeyer et al., 1973) can be used for absorption studies, however they do not allow the continuous control of tissue functionality like the Ussing chamber technique.

Gut epithelial cell culture models might help to understand local metabolism of xenobiotics in gastrointestinal cells, but although epithelial cell cultures from ruminal epithelium have been successfully established (e.g., Stumpff et al., 2009, 2011; Kent-Dennis et al., 2020), they have very rarely been used to analyze xenobiotic metabolism or transport. Most of the established ruminal epithelial cell culture models are based on primary ruminal cells harvested from freshly killed animals. Thus, the use of immortal cell lines to test drug metabolism and cytotoxicity (Allen et al., 2005) could reduce the number of required animals. Immortal enterocyte cell lines have been established from several species. For example, immortalized bovine epithelial cell lines from the rumen and small intestine have been used to study transport mechanisms of pathogens and to evaluate drug delivery via membranous epithelial cells (Miyazawa et al., 2010; Zhan et al., 2019). Another study established a sheep ruminal cell line to screen for possible interactions of transgenic feed and food compounds with the cells lining the rumen (Bondzio et al., 2008).

4 *In vitro* models for hepatic metabolism

Once nutrients or contaminants have crossed the intestinal epithelium, either via active transport, passive transcellular diffusion or the paracellular route, they are transported via blood to the liver or, when first transported with the lymph, enter the systemic blood circulation through the thoracic duct. Therefore, these substances may be subjected to different biotransformation and excretion pathways or may accumulate in different organs, including tissues used for human consumption. Understanding the fate of contaminants after intestinal absorption is thus pivotal for the assessment of risks for both animal health and consumer safety.

The liver, considered the most important metabolizing organ, consists of different cell types including hepatocytes, endothelial cells, stellate cells, Kupffer cells, pit cells, and bile duct cells. Hepatocytes account for approximately 80% of the healthy liver mass (Gerlach et al., 1994). The transporter-mediated uptake of endogenous substances and xenobiotics from the portal blood plasma into the hepatocytes occurs mainly via their sinusoidal (basolateral) membrane. In the context of metabolism, this process is referred to as phase 0 (Döring and Petzinger, 2014). In humans, transport proteins from the class of organic anion transporting polypeptides (OATP), organic anion transporters (OAT), cation transporters (OCT), and, to a lesser extent, the Na^+/taurochlorate cotransporting polypeptide (NTCP) are involved in the active uptake of compounds (Müller and Jansen 1997; Marin, 2012). In contrast to the state of knowledge in humans and exper-

imental animals, little is known about the occurrence of uptake transporters in ruminant tissues. It was shown that OATPs can also be found in cattle tissues (Liu et al., 2013; Xiao et al., 2014).

After uptake, hepatocytes are responsible for converting lipophilic (toxic) xenobiotics such as dietary and environmental pollutants (plant and fungal toxins, pesticides, herbicides, etc.) and drugs into water-soluble forms, so-called metabolites, which can be eliminated from the body via the excretory organs, i.e., kidney and intestine. However, this process can sometimes activate previously non-toxic substances into toxic metabolites or increase the potency of already toxic substances. Accordingly, the liver not only has a high detoxification capacity, but also an activating potential of xenobiotics (e.g., pyrrolizidine alkaloids; Mattocks, 1986; Roeder, 2000; Wiedenfeld and Edgar, 2011; or aflatoxins; Kuilman et al., 2000; Alvarado et al., 2017).

The two phases of biotransformation of both endogenous and exogenous compounds are referred to as phase I and phase II reactions. Phase I reactions are catalyzed by enzymes such as cytochromes P-450 (CYPs) or other oxidoreductases. Often, nucleophilic groups are introduced into the molecules, e.g., hydroxyl groups. Further phase I reactions are hydrations and dehydrations. Phase II enzymes such as uridine diphosphate glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), sulfotransferases (SULTs) or amino acid transferases conjugate nucleophilic groups of xenobiotics or their oxidized phase I metabolites with highly ionized hydrophilic molecules such as glucuronic acid (He et al., 2010; Maul et al., 2012; Li et al., 2017a), glutathione (Larsson et al., 1994; Muluneh et al., 2018), sulfate (Smith and Shelver, 2002; Li et al., 2017a) or amino acids (Knights et al., 2007). For this purpose, phase II enzymes require cofactors. Conjugates can be further metabolized or passed through an enterohepatic cycle. Excretion of phase I metabolites or conjugates can occur renally, biliarily, by sweat or by breath.

Species differences in metabolism between humans and animal species are of great importance for the risk assessment of xenobiotics. If the metabolism of compounds in humans and animals varies qualitatively or quantitatively, this leads to different concentrations of the given compound and its metabolites in the target organ or in the food of animal origin. Consequently, sensitivity to the compound also differs between the target species at the same administered dose. For example, of the 57 human CYP isoforms known to date, CYP1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5, 3A7 are attributed relevance for the metabolism of foreign substances. The isoforms mentioned have a low catalytic specificity, are often inducible and show considerable intra- and inter-species differences in their enzyme activities (Guengerich, 1997). Orthologous CYPs in the liver of humans, rats and ruminants can vary considerably with regard to their expression levels, substrate selectivities and catalyzed reactions (Pelkonen et al., 1998; Dacasto et al., 2005).

An *in vitro* model for the metabolism of contaminants should reflect the *in vivo* situation of the liver as closely as possible. Besides the perfused *ex vivo* liver described in Section 5.1, established *in vitro* models include liver slices (Viviani et al., 2017), primary hepatocytes (Ehrhardt and Schmicke, 2016; Witte et al., 2019), immortalized (transgenic) cell lines (Kuroda et al., 2015; Yoshioka et al., 2016), and subcellular fractions derived from liver homogenates. CYP, UGT or SULT isoenzymes can be expressed recombinantly in insect cells (supersomes) or *E. coli* (bactosomes) (Asseffa et al., 1989; Kost et al., 2005).

Subcellular fractions of liver tissue can be divided by differential centrifugation into the 9000 × *g* supernatant fraction (S9 fraction), cytosol and microsomes (von Jagow et al., 1965; Hubbard et al., 1985; Graham, 2002; Richardson et al., 2016). The S9 fraction, which is also commercially available from a variety of species, contains both the enzymes present in the cytosol (e.g., SULTs, GSTs) and those bound in the microsomes (e.g., CYPs, UGTs). After cell lysis, a solution containing broken cells, small fragments of the plasma membrane and the endoplasmic reticulum is obtained, while the organelles (e.g., mitochondria, nuclei, lysosomes) remain intact.

This lysate then can be subjected to a series of centrifugation steps in an ultracentrifuge to be fractionated into several components, e.g., into microsomes and cytosol (1 h at 104'000 × *g*). The microsomal fraction consists of vesicles that are formed from the endoplasmic reticulum, containing phase I and phase II enzymes (Kedderis, 2018; Sanchez and Kauffman, 2010). Microsomes are widely used to evaluate the metabolic stability and metabolite formation of a compound. Accordingly, they can be used to identify possible metabolization of undesirable substances that enter the animal after being absorbed from the gut. For this, the substance of interest is incubated with a buffer containing the microsomes and cofactors (Jia and Liu, 2007; Knights et al., 2016). The activity of the CYPs and UGTs, however, can vary depending on the buffer. In general, 50-100 mM Tris HCl (pH 7.5 at 37°C) or potassium or sodium phosphate buffer (pH 7.4) is suitable, but UGTs appear more active in Tris buffers than in phosphate buffers (Boase and Miners, 2002; Engtrakul et al., 2005; Argikar et al., 2016; Badée et al., 2019). Metabolism studies require the addition of cofactors for the target enzyme, e.g., 1-4 mM NADPH for CYPs or 2 mM uridine diphosphate glucuronic acid (UDPGA) for UGTs. The protein concentration of the enzyme solution can be adjusted depending on the application and susceptibility of the substrate (Jones and Houston, 2004). Metabolization can be started by preheating the substrate, buffer and cofactors at 37°C and adding the cold enzyme fraction. An alternative approach is to preheat the enzymes, substrate and buffer and start metabolization by adding the cofactor. The reaction is stopped at specified times by adding ice-cold organic solvent (e.g., methanol, acetonitrile optionally acidified with formic acid or ammonium formiate). The samples should then be well mixed by vortex and stored for at least 20 min at -20°C to -80°C to facilitate precipitation of proteins and salts, which can finally be sedimented by centrifugation (Jia and Liu, 2007; Knights et al., 2016). The enzymatic degradation of the substrate and the identification of metabolites may be investigated using, e.g., liquid chromatography coupled to mass spectrometry, nuclear magnetic resonance spectroscopy, UV or radioactivity detection (Wen and Zhu, 2015; De Vijlder et al., 2018; Foroutan et al., 2019; Zhang et al., 2019; Thiel et al., 2019; Tolonen and Pelkonen, 2015).

In vitro hepatic metabolism studies have been conducted for numerous contaminants (e.g., Maul et al., 2012; Kolrep et al.,

2018). Hayes et al. (1977) determined more than five phase I metabolites after incubation of aflatoxin B_1 with bovine liver microsomes. In a subsequent feeding study, one of these metabolites, aflatoxin M_1, was extracted using organic solvents from kidney, liver and mammary gland obtained from a cow receiving 7.31 mg/day aflatoxin B_1 over a 14-day period.

The metabolism of various toxic pyrrolizidine alkaloids present in many plants belonging to the families of *Asteraceae* has also been studied using liver tissue fractions from cattle (Huan et al., 1998; Duringer et al., 2004; He et al., 2010; Fashe et al., 2015; Kolrep et al., 2018; Muluneh et al., 2018). *In vivo* studies with cows provide evidence that some of these pyrrolizidine alkaloids or their metabolites are transferred to milk (Dickinson et al., 1976; Johnson, 1976; Candrian et al., 1991; Hoogenboom et al., 2011).

When the *in vitro* liver metabolism is known and the metabolites can be identified, it can be judged whether there is a need to measure these metabolites in food of animal origin (e.g., milk, meat). However, due to the often-lacking standards for the resulting metabolites, their discovery remains a challenging task.

In phase III of metabolism, the metabolites as well as unmetabolized substrates are transported out of the hepatocytes by a transporter-mediated efflux either via the basolateral membrane into the portal blood for renal excretion or via the canalicular (apical) membrane into the bile for fecal excretion (Döring and Petzinger, 2014; Müller and Jansen 1997; Marin, 2012). These transport proteins belong to the superfamily of ATP-binding cassette (ABC) transporters and are well characterized in humans. The data on the occurrence of efflux transport proteins in farm animals is as limited as on uptake transport proteins. Results exist on the expression of efflux transporter ABCG2/BCRP in tissue barriers of lactating dairy cows, sheep and goats (Lindner et al., 2013). The large data gaps regarding the transport mechanisms of xenobiotics in farm animal tissues have been pointed out by several scientists (Martinez et al., 2018; Virkel et al., 2019; Rosa, 2020). There remains an urgent need for research on this topic.

5 *Ex vivo* organ perfusion models

Biotransformation processes are the result of a complex interplay of different metabolic and cellular systems that cannot be captured with simple cell culture models. Excretion pathways via bile, urine or milk, putative re-absorption from the intestinal tract or further metabolism after initial hepatic biotransformation contribute to the complexity. Thus, *ex vivo* perfusion models including whole organs or even body parts may help to generate information about the fate of contaminants in the body without using live animals (Daniel et al., 2018). Organs may often be obtained from slaughterhouses instead of from purpose-killed animals (Grosse-Siestrup et al., 2002). Such perfusion models have a long tradition in toxicological testing and for studying and improving the preservation conditions for organs intended for transplantation in humans. They may also be used to gain insight into the metabolism and transfer of contaminants.

5.1 Kidney and liver perfusion models

The isolated and perfused liver already was described for various animal species almost a century ago (Plattner, 1924; Höber and Titajew, 1930; Haywood et al., 1945; Gunberg et al., 1955). The first model of a perfused bovine (calf) liver was described in the 1960s (Chapman et al., 1961). Studies using isolated perfused porcine and dog kidneys were conducted even earlier to study factors influencing urine formation (Loebell, 1849; Starling et al., 1925; Nizet, 1975). Today, studies are performed with porcine livers and kidneys from slaughtered animals due to their anatomical similarity to humans, their model role for circulatory death, and their accessibility (Grosse-Siestrup et al., 2003; Dondossola et al., 2019). The isolated porcine or bovine liver or kidney can be perfused with modified artificial fluids or heparinized blood and allows the study of the biotransformation of molecules of interest (e.g., contaminants), excretion through the venous blood, urine or bile, and possible adverse effects on hepatic or renal function. Sampling of arterial and venous fluids as well as bile allows toxicokinetic modelling at whole-organ level.

5.2 The perfused bovine udder

A variable amount of systemically available contaminants is excreted with the milk. This depends on their lipophilicity and degree of ionization, because the pH of milk (pH 6.5 to 6.7) is lower than that of blood. Therefore, partitioning of weak acids through the blood milk barrier is limited. Accordingly, the ratio of the ionized and the non-ionized form of compounds is high in the blood plasma.

The penetration of the blood-milk barrier by a compound can be predicted *in silico* based on the pH, pK-dependent partitioning phenomenon. Ziv and Rasmussen (1975), Shen-Tov et al. (1997) and others calculated the transfer rate of various compounds from blood plasma into milk and found a good correlation of the predicted data with results of *in vivo* studies (concentration in milk samples of treated cows). Possible inducible active transport processes should also be considered (Halwachs et al., 2013; Mahnke et al., 2016).

First perfusion models of the bovine udder already were described in the 1950s (Peeters and Massart, 1952; James et al., 1956; Verbeke et al., 1957). Since the isolated perfused bovine udder first was used to study the distribution of antibiotics in the udder tissue (Kietzmann et al., 1993), various *ex vivo* studies were performed to study the tissue distribution of β-lactam antibiotics, marbofloxacin and cefquinom (Ehinger and Kietzmann, 1998, 2000a,b, 2001, 2006; Kietzmann et al., 2008). Advantages of using the isolated perfused udder are that both tissue and milk samples can be taken repeatedly at various time points. A disadvantage is the relatively short duration of tissue viability of up to about 8 h, which must be controlled during perfusion. Suitable viability parameters that can be measured in the perfusate include glucose consumption, lactate production, and lactic dehydrogenase (LDH) concentration. Additionally, cell viability can be determined in tissue samples by cell viability tests. Another disadvantage is that isolated organs are deprived of nervous regulation and lymph drainage.

Medium sized udders of slaughtered healthy lactating cows are used. Directly after slaughtering, blood clots in the gland's vessels are cleared using heparinized Tyrode's solution. In the laboratory, the udder can be fixed in a "natural" position using a metal frame. The perfusion must be started within minutes after insertion of silicone tubes into the large arteries of each udder half. The large veins are also cannulated to allow sampling and removal of the perfusate. Smaller veins are closed using artery forceps. After an equilibration period, the perfusion is continued with a fluid containing the test compound. In most experiments by Ehinger and Kietzmann (1998, 2000a,b, 2001, 2006) and Kietzmann et al. (2008), the isolated bovine udder was perfused with Tyrode's solution, which resembles lactated Ringer's solution but contains magnesium, a sugar (usually glucose) as an energy source, and bicarbonate and phosphate instead of lactate. Perfusion with heparinized and diluted blood also is possible. However, selecting the most suitable perfusion medium depends on the solubility of the test compound in the perfusion fluid. When the time-dependent blood plasma concentration of a test compound or its metabolites is known, the udder perfusion can be performed with its adapted concentrations to simulate realistic situations. The measured tissue and milk concentrations allow calculating the amount of test compound that is eliminated via milk.

Unlike *in vivo* experiments on intact animals, the isolated perfused bovine udder model enables the investigator to retain control over various internal and external variables, such as type and composition of the perfusate or a certain treatment before or during the experiment, e.g., the impairment or improvement of organ physiology.

6 Integrating *in vitro* and *in silico* generated data using kinetic modelling

In vitro, *ex vivo* and *in silico* methods produce data about individual *in vivo* physiological processes describing the transfer of undesirable substances in ruminants. To integrate these data from individual methods into a coherent whole, a systems biology approach like kinetic modelling is required.

6.1 Toxicokinetic modelling

Kinetic modelling is a mathematical tool used to predict absorption, distribution, metabolism and excretion (ADME) of a substance of interest in live organisms. The substance may be a drug, in which case the term used is pharmacokinetics (PK), or a toxin or toxicant, in which case the term used is toxicokinetics (TK). Kinetic models describe the fate of a substance entering an organism by compartmentalizing the organism and using differential equations based on biochemical principles to describe the mass flows between various compartments and the chemical reactions happening within. These compartments may be ad-hoc groups of tissues and organs (Numata et al., 2014) or represent well-defined physiological structures, e.g., the extracellular space of the liver (Savvateeva et al., 2020). In the former case, the models are called compartment TK models, and in the latter case they are called physiologically-based toxicokinetic models (PBTK). Techniques exist to reduce the complexity incurred by including many physiological tissues and combine them into simpler compartment models, bridging TK and PBTK models (Pilari and Huisinga, 2010). Both flows and reactions in the compartments are represented with corresponding parametric differential equations, which yield the change in amount or concentration in each compartment over time.

Interest in PBTK models is growing, as their ability to accurately reflect the physiology of the underlying modelled processes makes them more accurate provided sufficient data is available. Modeling physiological processes may increase the predictive ability of PBTK models compared to empirical TK models. There is much potential in this field, and research is progressing fast, especially in the field of animal health (Lin et al., 2016). PBPK/TK models have been developed for ruminants (goats, cows) by Leavens et al. (2012) and Li et al. (2018). While the goat model by Leavens et al. (2012) was developed for veterinary purposes, the cow model was developed to ensure compliance with maximum residue levels of penicillin in milk.

In vitro-to-*in vivo* extrapolation (IVIVE) in conjunction with PBPK/TK is an approach to bridge *in vitro* and *in vivo* data and to examine the key mechanisms determining the kinetics. PBTK modeling has also been used to address IVIVE for animal-free risk assessment (Fabian et al., 2019). In that study, an eight compartment PBTK rat model was developed and its predictive efficacy assessed by comparing it to relevant *in vivo* studies.

Kinetic models can be built using available knowledge about physical and computational chemistry, animal physiology (models of systems), animal experimental data (empirical models) or a combination thereof (hybrid models) (Bonate, 2011). To describe the fate of a substance in an organism with (PB)TK modeling, the optimal kinetic equation parameters must be estimated, i.e., the parameters that ensure the model equations follow a time trajectory consistent with what one would observe *in vivo*. Data from *in vivo* feeding experiments can be used and fitted to the model equations to identify the kinetic parameters of interest (Bonate, 2011). Alternatively, one can use data derived from the literature, i.e., from *in vitro* models and from *in silico* prediction algorithms (computational toxicology) (Bolt and Hengstler, 2020) to obtain estimates for TK parameters, reducing or eliminating altogether the need for animal experiments (Paini et al., 2019). This approach poses challenges but at the same time has much potential (Lin and Wong, 2017), both for the sake of animal and human health and for saving time and money in the risk assessment of new undesirable substances. In the following, we present a summary of how (PB)TK models can be used as the final step to validate, complete and integrate the information gained from *in vitro* models of ruminants, such as the ones presented in the previous sections.

6.2 Integrating *in vitro* and *ex vivo* data using kinetic modeling

PBTK models are powerful tools that can be used to simulate the change in concentration of a xenobiotic in tissues (local internal exposure) of interest upon (external) exposure. They can be

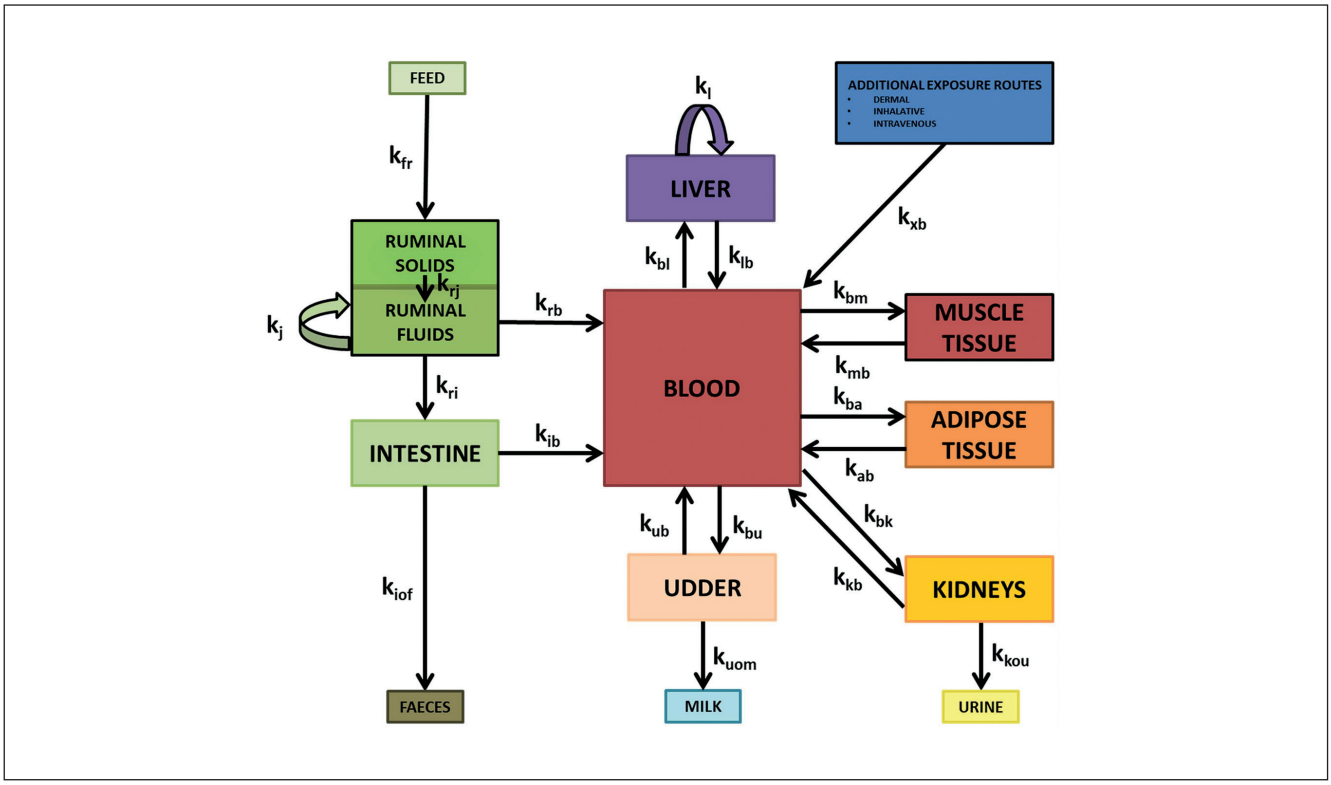

Fig. 1: A representative example of a toxicokinetic model for ruminants

The colored compartments represent the various organs or tissues; the flows of contaminants in and out of compartments are represented by straight lines and the chemical reactions happening in specific compartments by curved lines. These flows and reactions are governed by kinetic parameters, which are given next to the corresponding arrow. The concentration change of a contaminant (and potentially its metabolites) in each compartment is expressed in the form of a parametrized differential equation. One subscript letter: internal reactions (e.g., liver metabolization, microbiome-mediated reactions, etc.). Two subscript letters: mass flow from one compartment (first letter) to another (second letter). Three subscript letters: excretion mass flow from one compartment (first letter) followed by the "o" for output (second letter), followed by the excretion route (last letter). f, feed; x, exposure route; r, ruminal solids; j, ruminal fluid; i, intestine; b, blood; l, liver; m, muscle tissue; a, adipose tissue; k, kidney; u, udder; of, out feces; om, out milk; ou, out urine

extrapolated across species, doses, routes of administration and compounds, as well as used for IVIVE (Li et al., 2017b; Fabian et al., 2019; Lin et al., 2020). An example of how PBTK can be employed to extrapolate the fate of xenobiotics from dairy cattle to other species can be found in MacLachlan (2009). Such models require detailed knowledge on chemical attributes (e.g., partition coefficients) and, in the case of PBTK, of physiological variables (e.g., cardiac output, organ weights, and blood flow rates) (Lin et al., 2020), some of which can be challenging to obtain without *in vivo* data. In the next paragraphs, the feasibility of determining such essential variables using *in vitro*, *ex vivo* and literature data will be discussed for each of the organs and tissues most relevant for assessing the kinetics of xenobiotics in ruminants exposed through the oral route (Fig. 1).

Rumen

The rumen compartment is of crucial importance when modelling the fate of xenobiotics after oral intake, not only because it determines the rate of digesta flow to the intestine, but also because of the heterogeneity of gut microbiota, which may metabolize the modelled substance. The digesta flow can be estimated from physiological equations and animal science literature. Absorption into the blood can be estimated with data from Ussing chamber experiments. Predicting the chemical reactions that a substance may undergo requires a different approach. As discussed above, continuous or batch culture models can be used to investigate the metabolization of contaminants by ruminal microbiota. If reaction rates for metabolites can be obtained, they can be included in the model, improving its performance and accuracy.

Intestine

Two key parameters for the intestinal compartment are the absorption rate into blood and the rate of fecal excretion. The absorption rate from the intestine depends on a variety of factors, including the membrane's permeability, the substance's stability in the digesta, and the time available for absorption (Cho et al., 2014). The stability can be assessed by using incubation meth-

ods, as mentioned above. The time available for absorption is the transit time of digesta in the intestine, which can be found in animal science literature. As with the rumen, the permeability of the intestinal walls to a substance and epithelial metabolism can be investigated with the Ussing chamber technique. Fecal excretion rates can be calculated based on physiological equations and bioenergetics (Gabel et al., 2003). If the absorption rate of a substance into the blood and the frequency and amount of fecal excretion are known, substance concentrations in both feces and blood can be estimated.

Adipose, muscle and blood-tissue compartments

The blood-tissue compartment connects all other compartments in the (PB)TK model, reflecting the physical reality of the animal organism. The influx of substance from the GIT, which plays a significant role in determining the substance levels in blood, has already been described. Likewise important are the flows to and from the remaining compartments. Peripheral compartments are relevant for substances that accumulate in kinetically slower tissues (such as lipophilic substances in adipose tissue or lead in bones), and muscle tissue may be relevant for substances that can bind to proteins.

An effective approach in such cases is the estimation of partition coefficients. A partition coefficient is the ratio of concentrations of a compound between two distinct phases (in this case tissues or groups of tissues) at equilibrium. To apply partition coefficients, it is unnecessary that the transfer kinetics for all processes are in equilibrium or steady state, but it is crucial that the local distribution kinetics between the two relevant phases is fast compared to other kinetic processes (quasi-equilibrated state). The distribution of a substance from the blood to those tissues can be modelled as the diffusion between two phases of a solute, a process that may be adequately described by a partition coefficient. A range of such partition coefficient prediction algorithms exists, from those that use a simple octanol-water partition coefficient to account for the transfer of lipophilic substances into the tissue (Hermens et al., 2013) to more complex formulations, where the biological tissue is represented by a complex mixture of biochemicals (Schmitt, 2008; Poulin and Theil, 2000). Several *in vivo* and *in silico* methods are available to predict partition coefficients (Graham et al., 2012). More sophisticated predictors of partition coefficients, like those based on the polyparameter linear free energy relationship (pLFER) (Endo et al., 2013), can be used to capture the complex thermodynamics of biological molecules. Such an approach was applied by Savvateeva et al. (2020) to develop (PB)TK models of contaminants in growing pigs. Nevertheless, using partition coefficient prediction methods has limitations, given that they fail to represent differences in lipid types and other specific molecular interactions and non-covalent binding relevant for biological systems.

Specific binding to blood proteins can be studied using *in vitro* methods (MacManus-Spencer et al., 2010). For instance, serum albumin is the most important blood protein carrier for perfluoroalkyl acids (PFAAs) (Forsthuber et al., 2020). This binding can be explicitly built into PBTK models, where only the free, unbound fraction is available for exchange with other tissue compartments (Loccisano et al., 2013).

Liver

The liver plays an essential role in the metabolization of chemicals in the body. It can act both as a sink (detoxification) or as a source (bioactivation) of xenobiotics. It is therefore important to establish whether and how a xenobiotic is processed in the liver and to determine hepatic clearance (the rate at which a substance is degraded or transformed in the liver). Theoretical models that describe hepatic clearance are available for humans (Laveé and Funk, 2007) and can be adapted to other mammals such as ruminants. Information about the blood perfusion rate to the liver can be obtained from animal science literature (e.g., Lescoat et al., 1996). The actual liver reactions can be investigated either using *ex vivo* perfused liver models or *in vitro* hepatic models as described above. The liver biochemical reactions with their rate parameters can then be included in the PBTK model[1], encompassing possible self-induced metabolism (Savvateeva et al., 2020). Like other tissues, the liver may contain proteins that specifically interact with and store xenobiotics.

Kidneys

The kidney compartment in the model predicts the fraction of substance excreted via urine. Although no *in vitro* model exists that can simulate complete kidneys, the *ex vivo* perfused kidney model or *in silico* models can be used to obtain data without animal experiments. Kidneys are a challenging organ to model, since they are key not only to excretion processes but also to osmoregulation, which is subject to complex hormonal regulation and causes kidney activity to steadily change in response to internal and external stimuli. Nevertheless, it is possible to focus on physiological parameters of central relevance: glomerular filtration, tubular reabsorption and secretion. Depending on the modelled substance, reabsorption and secretion may be excluded from the model, whereas the glomerular filtration rate (GFR) is indispensable. In fact, the GFR has been shown to be proportional to renal drug clearance in some human PBPK renal models (Janků, 1993), making it one of the most relevant parameters for modelling renal excretion of xenobiotics. Although to date no such computational model exists for ruminants, the advances in predicting human renal clearance and urinal excretion (Doddareddy et al., 2006; Huang and Isoherranen, 2018; Watanabe et al., 2019) suggest the feasibility of developing similar prediction models for ruminants.

Udder

The udder compartment can be modelled by knowing the blood perfusion rate and the milk production rate, available in animal science literature (e.g., NRC, 1988), as well as other kinetic parameters quantifying the transfer through the blood-udder barrier

[1] Sontag, E. D. (2011). Lecture notes on mathematical systems biology. https://www.coursehero.com/file/41362918/Mathematical-Systems-Biologypdf/

(e.g., fraction of compound passing through the barrier). The latter can be experimentally investigated using the *ex vivo* perfused udder model by analyzing the perfusion fluid and the resulting milk equivalent for the substance.

6.3 Challenges and opportunities of using kinetic modeling to predict the transfer of contaminants into ruminant-derived food

There are still many challenges to using kinetic modelling to integrate the outputs of the many *in vitro*, *ex vivo* and *in silico* methods discussed to accurately predict the transfer of undesirable substances in ruminants to animal-derived food without the need for animal experiments. Although several studies could successfully generate predictive models for the transfer of xenobiotics in the animal body (Strikwold et al., 2017; Zhang et al., 2018, 2020; Cheng and Ng, 2017), a range of issues still must be addressed. The IVIVE represents a bottleneck in the development of models, and the estimation of model uncertainties may be difficult without independent datasets for evaluation and calibration. In some cases, industry-generated *in vivo* data, e.g., for pesticides and veterinary pharmaceuticals, can be used as validation datasets to avoid further animal experimentation. The search continues for alternatives to the comparison of model output with data from *in vivo* experiments as a validation approach. Another issue in the integration of *in vitro* generated data with *in silico* methods is the heterogeneity of the various methods' data output: This increases the complexity of the task and poses problems like the propagation of systematic and random errors in measurements, which can be difficult to estimate.

Despite the current issues around relying solely on mathematical modeling to predict the transfer of contaminants in animals, there are encouraging signs, as research is advancing rapidly, and the methods continue to improve. Not having to rely on animal experiments to predict the transfer of xenobiotics could speed up the risk assessment of new chemicals while reducing costs, contributing to both human and animal welfare.

7 Conclusions

Several of the here reviewed *in vitro*, *ex vivo* and *in silico* methods were established decades ago and are applied in laboratories worldwide. However, the number of studies centered on the transport and metabolism of undesirable substances in the ruminant animal is still modest. Although the applicable methods can adequately simulate individual body compartments, the challenge of aligning all their outputs to follow a xenobiotic and its metabolites of interest through the animal body remains. *In silico* approaches using toxicokinetic modelling offer the potential to integrate the data produced by a variety of methods and perform effective *in vitro* to *in vivo* extrapolation (IVIVE). The goal is to employ such methods to produce results comparable to *in vivo* experiments and to simulate diverse feeding and production scenarios. This represents a viable way to close the remaining gap between *in vitro* and *in vivo* results.

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Conflict of interest

The authors declare that they have no conflicts of interest.